HIGH FAT DIET CAUSES HEPATIC LIPID ACCUMULATION BY PROGRAMMING LIPID SYNTHESIZING GENES VIA GENE BODY METHYLATION

BY

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THESIS

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ABSTRACT

A high-fat diet (HFD) has been shown to cause more hepatic lipid accumulation as compared to a low-fat control (C) diet. However, it is still unclear what role epigenetic mechanisms play in hepatic lipid accumulation. We hypothesize that a HFD will cause greater hepatic lipid accumulation through increased lipid synthesizing gene expression via decreased gene methylation. The objective of this study is to determine the role of methylation in hepatic lipid synthesizing genes as an outcome of hepatic lipid accumulation due to a HFD. Two models were used to address the following: the direct effects of a post-weaning HFD on hepatic lipid synthesizing genes as well as the indirect effects of a maternal HFD. Timed-pregnant Sprague-Dawley rats were given either a C (16% fat) or HFD (45% fat) during gestation and lactation. Pups from both C mothers were weaned onto either a C or HFD and the pups from the maternal HFD were weaned onto a HFD, creating three offspring groups: C/C, C/HF, and HF/HF. Model 1 compared the C/C group to the C/HF group, while model 2 focused on the comparison of the C/HF group to the HF/HF group. Rats were sacrificed at 12 weeks of age, and the left lobe of the liver was used for further analysis. In model 1, liver histology showed greater hepatic fat accumulation in the C/HF group. Gene expression data was measured for multiple genes in the following pathways: glycolysis, gluconeogenesis, fatty acid synthesis, and triacylglycerol synthesis. Glycerol-3-phosphate acyltransferase (GPAM), fatty acid synthase (FASN), acetyl CoA carboxylase 1 (ACC1), and glucose-6-phosphotase (G6Pase) showed lower levels of mRNA in the C/HF group as compared to the C/C group. DNA methylation of those genes was analyzed based on data obtained from methyl-DNA immunoprecipitation with high-throughput sequencing (MEDIP-SEQ). Differential

methylation was observed for G6Pase, GPAM, and ACC1. GPAM and ACC1 showed decreased average methylation read peaks for C/HF in comparison to the C/C group. The data in model 1 suggest that decreased gene body methylation leads to decreased lipid synthesizing genes. In model 2, liver histology showed greater hepatic fat accumulation in the HF/HF group. Gene expression data were measured in the same pathways as previously stated. GPAM and FASN showed higher levels of mRNA in the HF/HF group. MEDIP-SEQ data showed greater average methylations read peaks for HF/HF along the gene body for GPAM. Methylation specific polymerase chain reaction (MSP) validated these results by showing greater methylation on intron 1, on the 7.2 kilobases (kB) pair region, as well as exon 21, at 59.5 kB pair region on GPAM. The data in model 2 suggest that increased gene body methylation leads to increased lipid synthesizing genes. These studies suggest that there is a positive relationship between gene body methylation and mRNA expression in hepatic lipid synthesizing genes.

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CHAPTER 1

INTRODUCTION

Obesity has increased more than two-fold since 1980 and comes with a multitude of complications and problems including non-alcoholic fatty liver disease (NAFLD) [20]. Part of the progression of NAFLD is the accumulation of fat in the liver, and a HFD has been shown to increase more fat accumulation in the liver as compared to a diet that is low in fat [25, 23, 22]. The accumulation of fat in the liver has been associated with increased hepatic lipid synthesizing genes such as fatty acid synthase (FASN1), glycerol-3-phosphate acyltransferase (GPAM), and acetyl-CoA carboxylase 1 (ACC1) [2].

However, the epigenetic mechanisms concerning gene body methylation that contribute to this increased gene expression are yet to be fully understood. Current research has shown that an increase in gene promoter methylation leads to a decrease in gene expression [10]. A HFD has been shown to upregulate lipid synthesizing genes such as GPAM, FASN, diglyceride acyltransferase (DGAT), and 1-acylglycerol-3-phosphate-*O*-acyltransferase 6 (AGPAT6) [25]**.** Furthermore, this increase in lipid synthesizing genes has been shown to increase due to early life exposure to a HFD [25]. In addition to a post-weaning HFD, a maternal HFD has been shown to increase fat accumulation [25]. A maternal HFD has also been shown to decrease gene promoter methylation in the triglyceride forming rate-limiting step enzyme; GPAM, in concordance with an increase in gene expression [6].

It is clear that a post-weaning HFD leads to increased hepatic lipid accumulation. In addition, early life exposure to a HFD, by maternal diet, programs lipid synthesizing genes via gene promoter methylation. However, it is not clear what the role of gene-body

methylation is in programming hepatic lipid synthesizing genes by the effect of a HFD as well as early life exposure to a HFD. In order to test the role of gene body methylation in differential gene expression due to a HFD induced hepatic lipid accumulation, we looked at two models. The first model that we looked at was how a HFD directly affects the gene body methylation status on various differential gene expression pathways. Due to the heritable nature of methylation, the second model we looked at was how a maternal HFD indirectly affects the gene body methylation status on the offspring's various differential gene expression pathways. These studies are the first to elucidate the epigenetic mechanism of gene body methylation in relationship to hepatic lipid synthesizing genes.

CHAPTER 2

LITERATURE REVIEW

The following is a literature review of recent research on the effects of a HFD on the epigenome by organ type.

Liver

It is clear that a HFD has an effect on methylation status in the liver. Zhang et al. looked at timed-pregnant Sprague-Dawley rats fed either a C or HFD (16% vs. 45% fat, respectively) was able to show that in rat hepatic tissue a HFD caused methylation differences. There were 12,494 differentially methylated regions (DMR) that were hypomethylated and 6,404 DMRs that were hypermethylated [23]. Even in cases where global DNA methylation is not changed a HFD seems to affect specific gene methylation [13]. Pacana et al. were able to show that in the absence of global DNA methylation a high fat and high cholesterol diet reduced DNA methyltransferase (Dnmt3a) in female C57BL/6 mice. HMG-CoA reductase, which is the rate-limiting enzyme in cholesterol production was found to be hypermethylated at CpG sites on the promoter. This would be expected as the high cholesterol diet would reduce the need for de novo cholesterol synthesis [13].

Promoter methylation of hepatic genes seems to be the most effected by a HFD. Also, a maternal HFD continues this effect into offspring [6]. Ehara et al. showed that in the livers of 5-day old mice pups whose maternal and paternal diets were a high fat and sucrose diet, expression of glycerol-3-phosphate acyltransferase (GPAT1) was increased. This increase in GPAT1 expression was associated with a decrease in its gene promoter methylation [6].

In addition to promoter methylation, a HFD affects hepatic gene expression via histone modification. Strakovsky et al. fed obesity resistant timed pregnant Sprague Dawley rats a HFD from embryonic day 2 until embryonic day 20 where they collected liver from offspring after caesarean delivery. The mRNA and pre-mRNA expression of phosphoenolpyruvate carboxykinase (*Pck1)* was higher in the liver of the HFD offspring. *G6Pase, Cebpalpha, Srebp1a and Pgc1a* mRNA content was higher in the HFD offspring. At the *Pck1* promoter, there was decreased H3Ac, H3K4Me2, H3K9Me3, and H3K27Me3 in the HFD offspring. In the coding region, there was increased H4Ac and H3K4Me2, but lower H3K9Me3 in the HFD offspring. In the 5 kB upstream region, there was increased H4Ac and H3K4Me2 in the HFD offspring [17]. While observing the male offspring, they saw significantly increased hepatic triglyceride content, but not in the female HFD group [18]. However, hepatic non-esterified fatty acid content was decreased in the HFD group offspring. Male HFD offspring had higher *Pon1* and *Pon3* mRNA expression. Both male and female had higher *Pon2* mRNA expression in the HFD offspring. There was also higher *Sod2* mRNA expression in male HFD offspring. However, the acetylation at H4 at the promoter for both male and female was increased. H3K4Me2 was also increased in male and female HFD offspring at the promoter [18].

Interestingly, Suter at al saw a decrease in H3K9Me3 and H3K14Ac in fetal liver collected at embryonic day 18.5 [19]. They looked at CD1 female mice, which were maintained on a C or HFD (9% vs. 35.5% respectively) for two weeks before mating with Glut 4 (G4) $^{+/-}$ and wild type males. They were on their respective diets throughout gestation and lactation. The offspring were weaned onto a low-fat diet (4.5% fat) at postnatal day 21. When looking at offspring at 5 weeks, this trend continued for the HFD

group. They saw that for the 5-week HFD group, Sirt 1 had significantly decreased; as Sirt1 deacetylates H3K14. Since they looked at CD1 wild type (WT) mice and Glut 4 knockouts, only the WT group showed a significant reduction in gene expression of Histone Acetyltransferase (Gcn) 5, Histone Deacetylase (Hdac) 1 and Hdac3. They performed ChIP-on-ChIP to determine the localization of H3K14Ac and H3K9Me3. They looked only at the fetal liver and observed robust enrichment in a broad region surrounding the transcription start site in each of the 8 groups that were studied. They used ChIP-on-chip to determine if there were any genetic differential enrichment between C vs. HFD. They saw 454 genes in the WT and 755 genes in the $G4^{+/-}$ that were altered significantly for both H3K14Ac and H3K9Me3. H3K14ac is enriched differentially in regions that contain Gata1, 2, and 4 and Myf5 binding motifs. H3K9me3 is enriched differentially in regions that contain GFY, E2F, E2F4, and RUNX-AML binding motifs. They generated gene lists using ingenuity pathway analysis (IPA) to determine which biologic networks are represented differentially. Four genes that are involved in lipid metabolism consistently emerged as central convergence nodes for the differentially represented pathways, which are: *Pparg, Ppara, Rora,* and *Rxra.* For each of these four genes, both the WT and $G4^{+/-}$ had H3K14ac and H3K9me3 enrichment in the HFD fetal liver. They used primers proximal to the TSS. Only *Pparg* mRNA was increased in the $G4^{+/-}$ HFD fetal liver. Indeed, H3K14Ac and H3K9Me3 are enriched in the promoter of the *Pparg* of only the $G4^{+/-}$ [19].

Furthermore, looking at the effects of a long term HFD on acetylation Mikula et al. looked at C57Bl/6J mice with a leptin gene mutation [11]. At six weeks of age, one group was fed a C diet (10% fat) and another a HFD (60% fat), until 16 weeks or 48 weeks.

Higher Tumor necrosis factor alpha (Tnfα) and Monocyte chemotactic protein 1 (Ccl2) mRNA in both 16 and 48 weeks in the liver of ob/ob and HFD-fed mice. Steatosis and mild inflammation were observed in the old HFD-fed mice and the young and old ob/ob mice. Neither polymerase II or NF-κB was increased for either Tnfα and Ccl2. In fact, in Ccl2 there was less pol II. The acetylation at H3K9K18 at the first and last exon of Tnfα was increased in the obese mice but not significant in Ccl2. The trimethylation of histone H3 lysine 4 (H3K4me3) is regarded as an indicator of active chromatin at the 5' ends of actively transcribed genes, but they saw no change of this in the obese mice for Tnfα. The first exon of Ccl2 had significantly less of this in obese mice, which is contrary to what you would expect based on the mRNA results. The trimethylation of histone H3 lysine 36 is associated with transcription elongation and is expected to gradually increase toward the 3' end of a transcribed gene. They saw no change in the Tnfα for obese mice, but mirrored the same results for H3K4me3 in Ccl2 for obese mice. They then looked into cell culture on mouse liver cells where they stimulated inflammation by treatment with Lipopolysaccharide (LPS). They then checked for the expression of Tnfα and Ccl2, which was increased. Then they checked for Pol2 levels in both exon 1 and the last exon and saw that it was increased for both genes. This was the same for Nuclear factor NF-κB. The peak expression of the pol II and NF-κB was at 90 minutes after LPS activation, and this was also the peak levels for H3K9K18Ac, at both the first and last exons, which is how they showed that the histone modification is causing the increase in Tnfα and Ccl2 gene expression. They were able to show that histone modification, mainly acetylation at H3K9K18 at the first and last exons, leads to increase gene expression due to the potential inflammatory effect of a HFD [11].

In contrast to an increase in acetylation, Yang et al. looked at the effects of a maternal high fat diet on the fetal liver [22]. They looked at 80 day old Sprague-Dawley rats, which were acclimated on standard chow for 1 week, before mating and going on either a HFD (45% fat) or a C diet (16% fat) throughout gestation and lactation. Rat livers were collected postnatal day 7. They saw decreased mRNA expression of *Wnt1* in HFD offspring as compared to C and decreased nuclear β-catenin protein in female HFD rats. They also saw a significant decrease in H4Ac at the *Wnt1* promoter in HFD rats, while in the coding region, there was a decrease in H3Ac and an increase in H3K9Me3 in the HFD rats [22].

Zhou et al. looked at Sprague-Dawley rats 10-12 weeks old, with either 16% fat or 45% fat throughout gestation and lactation [25]. After 21 days pups were weaned onto a C diet for 12 weeks before livers were collected. Interestingly, they saw many sex-specific differences. They saw an increase in the mRNA and pre-mRNA of *Pepck* in maternal HFD group for females but a decrease in males. FASN was increased in a female while Dgat and Agpat6 decreased. Gpam increased in male liver, while Cpt-1α decreased. They also saw an increase in transcription factor Cebpb in the female. Transcription factors Cebpa and Pgc1a decreased in male liver. Ash1l for H3K4Me3 and Suv39h2 for H3K9Me3 were increased for the maternal HFD group. In female maternal HFD group, promoter pol II binding was increased, and there was a significant decrease of H3K9Me3 but an increase in H3K27Me3. They plotted a line graph of different histone modifications while measuring protein abundance of pepck compared to changes along the 5' upstream to the 3' downstream region of pepck. For H3K4Me2 there was an increase at the -2kB, -1kB, +1kB, +3kB, +5kB, and +7kB. For H3K4Me3 there was an increase at +1kB and +3kB.

For H3K9Me3 and H3K27Me3 there was an increase at the promoter, while H3Ac was increased at +1kB. This data shows that both methylation of K4 and acetylation of H3 are positively associated with *Pepck* expression in a region-specific manner [25].

Cao et all was able to show that menin plays an essential role in hepatic lipid accumulation [3]. They bred menin knockout (KO) mice and showed that even at a young age, exposure to a C diet lead to 10% heavier livers in the menin knockout mice compared to the C mice. As the mice aged, hepatic lipid accumulation only accelerated, eventually showing a 40% increase as compared to the C mice. While analyzing hepatic genes related to lipid metabolism, they found that CD36, involved in fat storage, was the most significantly elevated. CD36 was also shown to increase in the livers of aging C mice. This intersecting point between fatty liver and aging was the foundation of their rationale to move forward into their next experiment where they bred $Men1^{flox/flox}$ mice with mice expressing the Cre recombinase driven by the albumin promoter. Heterozygous $Men1^{f/+}$-Cre^{+} mice were backcrossed with C57BL/6J mice 5 times, then crossed to generate hepatocyte-specific Men1 knockout mice ($Men1^{f/f}$ –Cre^{+}), with their C mice ($Men1^{f/f}$ –Cre^{-}). After 8 weeks, mice were then fed on their respective diets for 14 weeks (HFD vs C). They used HEK293T cells to show that endogenous menin could be pulled down by SIRT1 or vice versa. Using truncated flag-menin constructs to map the binding sites of SIRT1 they showed that the N-terminus, C-terminus, leucine zipper-like motif deletions and the middle part of menin could bind to SIRT1. This is important as SIRT1 is a primary histone deacetylase. If menin typically recruits SIRT1 to the promoter of CD36 to down-regulate its expression then losing this regulation may lead to over-acetylation on the promoter of CD36 thus leading to the overexpression of CD36. In order to check for this,

they isolated primary hepatocytes from the menin KO and C mice and used ChIP to look at the promoter region of CD36, in which they used 7 different primers that cover around 400 bp each on the promoter. In the C mice, they saw that the CD36 promoter had a binding of SIRT1 and menin. In the menin knockout mice, they saw that the 1710-1246 bp, 1338-875 bp, 1425-1085 bp, and 568-166 bp of upstream regions of the promoter showed reduced SIRT1 binding and increased H3 acetylation. Their mechanism showed that the loss of menin lead to increased CD36 expression since SIRT1 was not brought to the promoter by menin. This data shows that hepatic lipid accumulation may occur by increased promoter acetylation of CD36 due to the HFD reducing menin expression and SIRT1 recruitment [3].

These data show that a HFD can affect histone modification by increasing or decreasing acetylation and methylation. However, whether it is increased or decreased directly varies along different genes, gene location, and regulatory pathways. Although the data does not reach a clear consensus on the direction of the effects of a HFD on histone modification, it is clear that histone modification is mechanistically used to affect hepatic gene expression.

Adipose Tissue

Multhaup et all looked at the methylation status in adipose tissue [12]. They examined male C57BL/6 mice at 4 weeks of age that were fed either a HFD or a C diet for 12 weeks. Using comprehensive high throughput array or CHARM, 232 differentially methylated regions (DMRs) correlated with diet status. They found hypermethylation on the promoter of Phosphoenol-pyruvate carboxykinase 1, a rate limiting enzyme in gluconeogenesis. The genome wide DMRs were near genes that were significantly

overrepresented in lipid metabolism and immune/inflammatory pathways. Interestingly, the DMRS that were hypermethylated seem to be near enriched genes of metabolism, while the hypomethylated regions were near enriched genes of inflammation. They saw a trend of an inverse correlation of gene expression to gene methylation. Out of 625 significant mouse adipocyte DMRs, 576 of those DMRs had homologous regions in the human genome, and 497 of the DMRs were conserved. Of the 497 conserved DMRs 249 of those were significantly different in obese vs. lean humans. They also showed that the main mechanism for methylation-mediated metabolic dysfunction is likely through epigenetic enhancer modification. They showed that 40.6% and 14.7% of the directionally consistent 170 regions and 53.3% and 20% of the further 30 genome wide associated study for type 2 diabetes-associated regions were positioned in adipose enhancers and super enhancers, respectively [12].

These changes in methylation status in mouse adipocytes lead them to check human adipocytes. They utilized cell culture of samples from 7 lean, 16 obese and 8 obese post Roux-en-y gastric (RGYB) subjects to look at the effects of gene regulation implicated in obesity, type 2 diabetes, and insulin resistance. They looked at 5 genes that they saw had methylation reversal from RGYB bypass surgery, but that had no prior association with metabolic phenotypes. They then either upregulated or knock-downed these genes to mimic hypo and hyper methylation in high fat fed adipocytes, respectively. 4 out of the 5 genes that were either knocked out or over-expressed significantly altered glucose uptake. This shows that although these genes have not previously been associated with metabolic phenotypes, the method to look at methylation changes in concordance with phenotypic changes due to interventions like RGYB bypass surgery,

may be a good way to understand the mechanism of how a HFD can cause insulin resistance, obesity, and type 2 diabetes [12].

Xia et al. looked more specifically at promoter methylation for the gene leptin [21]. They looked at male C57BL/6J mice at 3 to 4 weeks of age, which were given either a 60% fat HFD or a 10% fat C diet up to 18 weeks. They were able to establish 16 CpG sites in the leptin promoter in c57 mouse epididymal fat. They found, on average, more methylated promoter DNA at 12 weeks and 18 weeks of a HFD compared to the earlier weeks and the C diet group methylation; thus, leading to reduced leptin production from adipose tissue [21].

Not only does methylation affect the promoter of leptin in adipose tissue due to a HFD but Shen et al. was able to elucidate a role of acetylation in this pathway [15]. They used 3 to 4-week-old C57BL/6J mice and put them on three different diets (HFD 60% fat kcal, HFD 60% fat w/ n-3 PUFA, and LFD 10% fat kcal) after 1 week of acclimation. They stayed on their respective diets for 14 weeks. Interestingly, they saw higher levels of plasma leptin in the HFD mice compared to C. 9 out of 16 CpG sites in the leptin promoter were more methylated in the HFD mice compared to the C, which seems to contradict the direction of the current literature. Three of those CpG sites are specific for the binding of the transcription factors SP1 and Lp1. There were significantly higher levels of DNA Methyl-transferase (DNMT) 1, DNMT3a, DNMT3b, and MBD2 at the leptin promoter in the HFD mice. Naturally, they saw less pol II binding at this promoter. At the promoter of leptin, there was significantly fewer levels of H3Ac, H4Ac for the HFD group. It is interesting to note that compared to Cao et al.'s work this is in direct contradiction as they showed increased acetylation on the CD36 promoter leads to increased expression of it;

here Shen et al. shows the opposite effect [3, 15]. There was also the higher binding of HDAC1, HDAC2, and HDAC6. They saw significantly fewer levels of H3K4Me at the leptin promoter for the HFD group. The average of the methylation for the 16 CpG's on the promoter of leptin proved to be less at 8 weeks but higher at 12 and 18 weeks in the HFD diet group. They also showed two correlation graphs: Increased mRNA expression of leptin was correlated with increased promoter methylation in the HF group and decreased mRNA expression of leptin in C group was correlated with decreased promoter methylation [15].

Fan et al. used a HFD mouse model to analyze DMRs [7]. They showed that 24 promoters were hypermethylated and 42 were hypomethylated in epididymal fat tissue that overlapped with transcriptionally expressed genes. They also showed that with genes that have high CpG sites within the promoter, a negative correlation exists between transcriptional expression and methylation. However, they did not see this correlation with genes that have an intermediate or low amount of CpG sites within the promoter. They saw that the genes that were hyper-methylated and under-expressed were relevant to metabolic processes such as lipid metabolism, type 2 diabetes, obesity linked pathways, etc. The reverse of that was also true; genes that were hypomethylated and overexpressed also were linked to metabolic processes. In the three genes examined, Mmp2, Foxj3, and Ube2q2, increased average promoter methylation levels were found in Foxj3 and Ube2q2 but significantly less in the Mmp2 promoter. The gene expression data showed an inverse relationship with promoter methylation [7].

Interestingly, although most studies show the inverse relationship between promoter methylation and gene expression, with Shen et al.'s study seeming to be an

outlier; Gracia et al.'s study may provide some corroboration in support of Shen et al.'s study [8, 15]. They looked at the adipose tissue of male Wistar rats fed an obesogenic diet at 45% fat. When observing the promoter of FASN in the HFD group, they saw hypomethylation in the -90bp position and hypermethylation in the -62bp position compared to C diet rats. This pattern of methylation resulted in a fivefold increase gene expression for the HFD after 6 weeks. This is a case where different positions within the promoter have different outcomes on gene expression. It is possible that the -90 bp CpG position has a more relevant role in the expression of FASN or as Shen et al.'s study suggest; a role for methylation in the promoter for increased gene expression [8, 15].

Brain, Blood, Muscle & Colon

Cifani et al. examined male Sprague Dawley rats fed a HFD with either obese resistant (DR) or obese prone (DIO) groups [5]. They looked at promoter methylation in NPY, PPARy, and POMC in hippocampal tissue. On the NPY promoter, they found an increase in methylation on the 5^{th} CpG site for the DR group, which carried its effect into transcriptional gene expression as there was a significant decrease in NPY mRNA. They found increased methylation on POMC promoter at 21 weeks in the DR group compared to the DIO group. This is interesting due to the anorexigenic properties of the POMC gene, as we would expect to see less promoter methylation in the DR group. However, despite increased promoter methylation, they did see increased mRNA levels of POMC. In this study, increased promoter methylation seems to be positively associated with an increase in the mRNA levels of POMC, yet negatively associated in the case of NPY [5].

Zheng et al. looked at a mouse model with a maternal high fat and high sucrose (FS) diet with either a FS or C diet in the offspring. Maternal FS combined with FS

offspring diet showed increased expression of POMC and MC4R in the hypothalamus. Looking at DNA methylation in the hypothalamus, differential methylation patterns emerged between FS-FS and C groups. There was hypomethylation of the POMC promoter in the high-fat offspring which is positively associated with POMC gene expression. However, methylation did not seem to be the epigenetic mechanism that explained the increased expression of MC4R. This study seems to follow the trend in the literature showing an inverse relationship between promoter methylation and gene expression and in direct contradiction to Cifani et al.'s study showing a positive relationship between POMC promoter methylation and gene expression [5, 24].

Marco, Kisliouk, Tabachnik, Meiri & Weller raised Wistar female rats on either a HFD or a C diet and at post-natal day (PND) 80 they sacrificed half the rats and bred the other half with male Wistar rats [9]. After pregnancy at post-natal day 22, they sacrificed the dams. They also sacrificed female pups at PND 22 and then raised the rest until PND 80 on a C diet. At PND 80 they sacrificed females and put the rest of the rats on a 30 day HFD. HFD-C pups weighed more at P22 and ate more kcals/day. The HFD-C compared to the C-C group weighed more throughout their lifetime and weighed more during the 30 day HFD challenge that was presented at the end of their trial: HFD-C-HFD weighed more than the C-C-HFD. In the nulliparous female rats, although they were higher in body weight after 2 months of HFD they did not have higher levels of pomc mRNA. Despite having more leptin, their pomc/leptin ratio was still lower compared to the C diet group. They found more methylation at three specific sites on the *Pomc* promoter in the HFD group as compared to the C group. At the end of lactation, they saw a weight decrease in both groups, but the HFD group still weighed more. This loss in weight was seen in

concordance with both groups showing similar levels of pomc and leptin mRNA. The promoter methylation of pomc between both groups was similar. This methylation data was similar to Cifani et al in that there was increased POMC promoter methylation in the HFD group [5, 9]. However, this did not correspond to increased gene expression. When they looked at the hypothalamic arcuate nuclei of the HFD group, they saw that POMC promoter hypermethylation was correlated with decreased POMC expression. Here, the inverse relationship of the POMC promoter methylation to gene expression is further established [9].

Sanchez, Reynoso-Camacho & Salgado looked at the blood of male and female Wistar rats fed either a HF and high fructose diet or a C diet [14]. They crossed $Male_{HFD}$ x $Female_{HFD}$, $Male_{HFD}$ x $Female_{Control}$ and $Male_{Control}$ x $Female_{HFD}$ at two different time points (24 & 36 weeks), which then were divided between the two different diets. They looked specifically at global methylation in peripheral blood leukocytes of the parental animals. When looking at the change in methylation over time, they saw that between the males and females there were differential responses. In males, the HFD induced methylation that was lower compared to C, and although methylation increased over time, it was always lower than the C group. In females, a HFD induced higher methylation as compared to their C counterparts. They then looked at the whole blood of the offspring (F_1) and saw that compared to their parents (F_0) only the females fed the C diet showed increased methylation as compared to the F_0 at the 12-week time point. Other than that, there were no differences between generations. They concluded that there was a positive association between methylation and metabolic syndrome symptoms [14]. Although they were not able to establish any specificity in intragenic methylation this does provide

evidence that increased global methylation may be an indicator of metabolic dysregulation.

Amaral et al. looked specifically into mouse gastrocnemius tissue of mice fed a HFD, omega-3 supplementation or both [1]. Hepatic peroxisome proliferator-activated receptor gamma transcript variant 2 (Pparg2) mRNA increased due to a HFD and decreased with fish oil supplementation. Although the HFD group had almost a 6-fold increase in Pparg2, promoter methylation showed no differences. In the gastrocnemius muscle, the HFD group showed increased global DNA methylation along with decreased Pparg2 gene expression. Interestingly, when looking at promoter methylation for Pparg2, there was decreased methylation at the -247 bp CpG. Interestingly, these data showed a positive correlation with promoter hypomethylation and decreased gene expression [1].

Choi, Tammen, Liu & Friso examined how a HFD vs. aging affects global DNA methylation patterns [4]. In mouse colon, they found decreased DNA global methylation patterns due to a HFD but not due to aging. They observed promoter specific DNA methylation changes due to both effects, but only the HFD caused a global DNA methylation response. Of importance, the p16 promoter had more methylation in both aging and HFD groups. This is of significance since the p16 promoter is commonly methylated in colon cancer cases. This study corroborates Sanchez, Reynoso-Camacho & Salgado's study in that HFD increases global methylation that leads to deleterious effects [4, 14].

After thoroughly reviewing the effects of a HFD on epigenetics in multiple organs; the majority of the studies seem to establish an inverse relationship between promoter methylation and gene expression. However, there were a few studies of interest that

showed an opposite effect and may provide some evidence to a possible positive correlation between promoter methylation and gene expression in certain genes or CpG sites. Although a clear pattern in gene expression directionality was not confirmed for histone modification, it is clear that a HFD affects histone modification with the outcome of differential gene expression. Lastly, it is important to note that although we saw much promoter methylation data and a few studies on global methylation data, it is unclear what the effects of gene body methylation have on gene expression as well as how a HFD might affect gene body methylation.

CHAPTER 3

MATERIALS & METHODS

Animals

Timed-pregnant Sprague Dawley rat dams (Charles River Laboratories, Wilmington, MA) were fed either a standard AIN93G C diet (Research Diets, Inc.; 16% calories from fat) or a diet high in fat (HF; Research Diets, Inc.; 45% calories from fat) during gestation and lactation. Dams were individually housed with their pups in standard polycarbonate cages in a humidity- and temperature-controlled room on a 12-hour light-dark cycle with *ad libitum* access to food and drinking water [23]. On postnatal day 21, male offspring were divided into three groups: one group was kept on the same C diet (C/C, n=7) as C diet dams, the other group was kept on the same HFD as the HFD dams (HF/HF, n=8), and the last group switched to a HFD from the C diet dams (C/HF, n=8) (Figure 3-1). Male offspring were fed their respective diets for 9 weeks, at which time animals were sacrificed and the median lobe of the liver was immediately frozen in liquid nitrogen and stored at -70°C. Institutional and governmental regulations regarding the ethical use of animals were followed during the study. The protocol for ethical use of animals for this study was approved by the University of Illinois Institutional Animal Care and Use Committee (IACUC protocol no. 09112).

Liver Histology

Frozen liver samples comparing C/C and C/HF were embedded in Tissue-Tek OCT compound (VWR, Radnor, PA, USA) and cut to a thickness of 7 μm in a cryostat at -20°C for model 1 and 5 μm for model 2. All sections were fixed in 10% ice cold formalin for 5 minutes and then stained with Oil Red O (ORO) solution. Images were obtained by

the NanoZoomer Slide Scanner and NDP View software (Hamamatsu, Bridgewater, NJ, USA). ORO images were quantified using ImageJ software and statistically compared with standard T-Test on Microsoft Excel.

Frozen liver samples comparing C/HF and HF/HF were embedded in Tissue-Tek OCT compound (VWR, Radnor, PA, USA) and cut to a thickness of 5 μm in a cryostat at −20°C. All sections were fixed in 70% ethanol and then stained with hematoxylin and eosin (H&E) or Oil Red O (ORO) solution. Images were obtained by NanoZoomer Slide Scanner and NDP View software (Hamamatsu, Bridgewater, NJ, USA). All images were analyzed by two unbiased observers who were blinded to the identity of the samples. The observers were asked for their input on intensity, and the figures show representative images of samples from both groups. The fatty liver damage scale was used to quantify liver damage and ranges from 1-5, with healthy liver scored between 1.5-2.

MEDIP-SEQ and MRE-SEQ

Genomic DNA isolation was performed according to previously published methods [23]. Next generation sequencing was performed on one animal from each of the C/C, C/HF, and HF/HF groups. Gene expression and histology were measured in each animal, and the best representative from each group was chosen for sequencing. Methylation was measured using methylated DNA immunoprecipitation (MEDIP-SEQ) and methylation-sensitive restriction enzyme (MRE). MEDIP-SEQ utilizes antibodies against 5mC to quantify methylated DNA sequences while MRE-seq uses restriction enzymes that cut at unmethylated CpG sites. Run together, MEDIP-SEQ and MRE-SEQ improve coverage and resolution [32, 33]. The laboratory of Dr. Ting Wang at the University of Washington St. Louis, MO used previously established protocols to run the high

throughput sequencing [34,35]. Subsequently, bioinformatics analysis was performed in Dr. Yuan-Xiang Pan's lab at the University of Illinois.

DMR identification

All MEDIP-SEQ sequences were aligned to the rn4 rat genome assembly with Burrows-Wheeler Aligner (BWA) and processed using methylQA [31]. Average methylation read peaks were selected by the following criteria: ≥ 50% higher methylation increase and or opposite slope directionality in concordance with ≥ 50% higher methylation increase.

Methylation Validation

Validation of DNA methylation was performed on hepatic tissue from all animals using methylation-specific PCR (MSP). Genomic DNA was isolated from hepatic tissue using the DNeasy Tissue Kit (Qiagen) and was treated with sodium bisulfite reagent using the EZ Methylation- Gold kit (Zymo Research). Bisulfite converted DNA samples were diluted to 10 ng/ul for quantitative PCR analysis. A standard curve was generated by pooling two 20 ng/ul DNA samples from each group performing a 1:2 serial dilution for a total of 8 points. Primer Express Software 3.0.1 (Applied Biosystems) was used to design primers for quantitative PCR. Four primers were designed for each DMR of interest. Forward and reverse primers amplified the bisulfite converted DMR sequences that were either methylated (containing protected CpG) or unmethylated (containing thymines in place of all cytosines). Primers had a melting temperature (Tm) of 58-60 °C and contained minimal predicted hairpins, self-dimers, and heterodimers. Primers were between 22 and 38 nucleotides, and the total amplicon size was between 72 and 225 bp.

The PCR was performed using a 96-well plate with a final reaction volume of 10 µl. Each reaction contained 5 µl of Power SYBR® Green Master Mix (Life Technologies), 2.2 µl of nuclease-free water, and 0.4 µl of 5 µM each of the forward and reverse primers. The reaction was as follows: 95 °C for 15 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min (Applied Biosystems, StepOnePlus real-time PCR system). The relative amounts of methylated and unmethylated DNA were analyzed based on their respective standard curves. Percentage of methylated DNA was calculated with the following equation: % of methyl DNA= (relative quantity of methylated DNA) / (relative quantity of methylated DNA + quantity of unmethylated DNA)

Gene expression

Total RNA was obtained using 50 mg of liver tissue digested in TRIzol® Reagent (Life Technologies) followed by Direct-zol™ RNA MiniPrep columns (Zymo Research) with in-column DNase I digestion for RNA isolation. RNA concentration and purity were measured using Nanodrop 2000 (Thermo Fisher Scientific). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and incubated in the 2720 Thermal Cycler (Applied Biosystems) using the following protocol: 25°C for 10 min, 37°C for 2 h and 85°C for 5 s. Following reverse transcription, a standard curve was generated by serial dilution of four pooled samples. cDNA samples were amplified using Power SYBR® Green Master Mix (Life Technologies) in a StepOnePlus™ Real-Time PCR System (Life Technologies). The inclusion of a melting curve ensured purity of the PCR product. All reported mRNA quantities are normalized to a geometric mean of *Actb* and *Rpl7a* gene expression.

Chapter 4

HIGH FAT DIET CAUSES HEPATIC LIPID ACCUMULATION BY PROGRAMMING LIPID SYNTHESIZING GENES VIA GENE BODY METHYLATION

Results

Model 1

Physiological Observations

Liver Histology

Hepatic lipid accumulation tested by Oil Red-O staining showed greater hepatic lipid accumulation in the C/HF group as compared to the C/C group. Percent area measured between the two groups showed a significantly higher percentage of area covered by fat in the C/HF group at around 17.5% compared to only 7% in the C/C group (Figure 4-1).

Gene Expression

Gluconeogenesis (GNG)

We did not see any differences in PEPCK, but we did see a decrease in G6pase in the C/HF group as compared to the C/C. We did not see any differences in FBP1 (Table 4-1).

Fatty Acid Synthesis

When looking at the fatty acid synthesis pathway, we did see multiple genes differentially expressed. Specifically, in the C/HF group, both ACC1 and FASN were decreased when compared to the C/C group (Table 4-1).

Triacylglycerol Synthesis

Furthermore, we saw an increase in the lipid storage gene GPAM but not AGPAT6 or DGAT1 in the C/C group (Table 4-1).

Methylation

MEDIP-SEQ

When looking at the MEDIP-SEQ data for ACC1 and GPAM, we see that there is indeed differential methylation between C/C and C/HF (Figures 4-2 – 4-7). Unlike previous studies, the mRNA expression data were not correlated with the promoter methylation pattern of both ACC1 and GPAM as we did not see any significant differences between the groups. However, in the absence of differential promoter methylation, we did see 12 greater average methylation read peaks along the gene body of ACC1 in the C/C group with only three greater average methylation read peaks for the C/HF group (Figures 4-2 – 4-5). We see that at the following positions along the gene body of ACC1 there was greater average methylation read peaks for the C/C group: +13.5 kB, +14 kB, +26 kB (Exon 5), +33 kB, +33.5 kB (Exon 8), +65.5 kB, +68.5 kB, +75.5 kB (Exon 24) +76.5 kB (Exon 25), +133.5 kB (Exon 40), +153 kB, +170 kB, and +194 kB (3' Downstream Region) (Figures 4-3 – 4-8). We saw that at the following positions along the gene body of ACC1 there was greater average methylation read peaks for the C/HF group: +117 kB, +122 kB and +127 kB (Figures 4-2 – 4-5). Comparatively, the data suggest that the C/C group has greater overall methylation along the gene body and 3' downstream region. When looking into the methylation patterns along GPAM, we saw a similar trend compared to the methylation pattern we observed along ACC1. There are no differences in promoter

methylation; however, we see seven greater average methylation read peaks for the C/C group and only two greater average methylation read peaks for the C/HF group (Figures 4-6 – 4-7). We saw that at the following positions there was greater average methylation read peaks for the C/C group: +4 kB, +10.5 kB, +22 kB, +28 kB, +32.5 kB, +40 kB (Exon 8), and +43 kB (Exon 9). We saw that at the following positions there was greater average methylation read peaks for the C/HF group: +5 kB & +47.5 kB (Exon 13) (Figures 4-6 – 4-7). The methylation pattern along the gene body of GPAM follows the same pattern that we see in ACC1, with greater overall gene body methylation.

Model 2

Physiological Observations

Liver Histology

Oil Red-O staining showed greater hepatic lipid accumulation in the HF/HF group as compared to the C/HF group. The liver structure was tested using H&E staining and showed greater hepatic vacuolation in the HF/HF group as compared to the C/HF group (Figure 4-8). Comparing gradation on the fatty liver damage scale the C/HF group scored a 3, while the HF/HF group scored a 4.5.

Gene Expression

Fatty Acid Synthesis

When comparing the C/HF group to the HF/HF group, we observed more FASN in the HF/HF group (Table 4-1).

Triacylglycerol Synthesis

When considering the comparison between the C/HF group and the HF/HF group we saw a similar pattern in the lipogenesis trend in model 1 as GPAM was differentially expressed (Table 4-1). However, in this case the group that had greater hepatic lipid accumulation, HF/HF, had the increase in mRNA expression.

Methylation

MEDIP-SEQ

When looking at the MEDIP-SEQ data for GPAM between C/HF and HF/HF, it is clear that we see differential methylation between the two groups. Similar to the first model, we see no differences in promoter methylation but rather along the gene body. The HF/HF group has greater gene body methylation overall, as we see four separate greater average methylation read peaks and only one peak greater in the C/HF group. We see that at the following positions along the gene body of GPAM there was greater average methylation read peaks for the HF/HF group: +10.5 kB, +12 kB, +41 kB, and +56.5 kB. In comparison, only the following two peaks showed greater average methylation reads for the C/HF group: +10 kB and +53.5 kB (Exon 19) (Figures 4-9 & 4-10). These data suggest that in the HF/HF group there is greater gene body methylation.

MSP

In order to validate the MEDIP-SEQ data, we used MSP to check for methylation at specific locations along the gene body and 3' downstream region. We checked six different locations along the gene body and 3' downstream region, and although all six locations showed greater levels of methylation for the HF/HF group, the following two

locations showed significantly higher levels for the HF/HF group: Intron 1 +7200 kB and Exon 21 +59500 kB (Figure 4-11).

Discussion

In these studies, we attempt to understand how methylation is involved in the gene expression of hepatic lipid synthesizing genes due to hepatic lipid accumulation by a HFD. To investigate the role of methylation we looked at two different hepatic lipid accumulating models. The first model we looked at was the effects of a post-natal HFD on the methylation status of hepatic lipid synthesizing genes. Male Sprague-Dawley rats were exposed to either a HFD or C diet for nine weeks. We then collected liver samples and ran qRT-PCR to check for differences in multiple metabolic pathways including glycolysis, gluconeogenesis, fatty acid synthesis, and triacylglycerol synthesis. We found differences in mRNA expression for the gluconeogenic, fatty acid synthesis and the triacylglycerol synthesis pathways. Of those pathways, there were four major enzymes that we found to be differentially expressed. For the gluconeogenic pathway, the enzyme G6Pase was decreased in the C/HF group. For the fatty acid synthesis pathway, we found two enzymes that were decreased in the C/HF group: ACC1, which is the rate-limiting step in fatty acid production and FASN. Lastly, in the triacylglycerol synthesis pathway, we found the rate-limiting enzyme, GPAM, to be decreased in the C/HF group. Upon further inspection of the methylation status on ACC1 and GPAM, we found differential methylation patterns along the gene body. In order to more comprehensively understand the role of methylation, we looked into a second model, which addresses the heritability of methylation. The second model we looked into were the effects of a maternal HFD on the methylation status of hepatic lipid synthesizing genes. Timed-Pregnant Sprague-

Dawley rats were exposed to either a HFD or C diet during gestation and lactation. The pups were weaned onto a HFD for 9 weeks. We then collected liver samples and ran qRT-PCR to check for differences in multiple metabolic pathways including glycolysis, gluconeogenesis, fatty acid synthesis, and triacylglycerol synthesis. We found differences in mRNA expression in both the fatty acid synthesis pathway and the triacylglycerol synthesis pathway. The two major enzymes that we found to be differentially expressed were FASN and GPAM. Upon further inspection of the methylation status on GPAM, we found differential methylation patterns along the gene body.

In order to store fat in the liver, two major steps need to occur: fatty acid synthesis and triacylglycerol synthesis. ACC1 is the rate-limiting enzyme that converts acetyl-CoA into malonyl-CoA, which is the building block used to create fatty acids by the FASN enzyme. Through a serious of steps that involve GPAM, which is the rate-limiting enzyme in triacylglycerol synthesis, long-chain acyl-CoA is transferred onto a glycerol-3-phosphate backbone eventually creating triacylglycerol [28]. Overexpression of these enzymes has been linked to hepatic lipid accumulation [2]. Since hepatic lipid accumulation is limited to either an increase in fatty acid and triacylglycerol synthesis or a decrease in beta oxidation or lipid export the mRNA expression data showed evidence of a possible physiological enzymatic mechanism. Since the mRNA expression data did not show any differences in the lipid export or beta oxidation pathways and the differential mRNA expression data shown in the fatty acid and triacylglycerol pathway show the decrease to occur in the C/HF group, the overload of dietary lipid influx is the most plausible explanation for the hepatic lipid accumulation. In the first model, we are working with mice that have presumably normal hepatic physiology as the maternal diets were

both C, therefore; it is not surprising that an intervention with a HFD would downregulate *de novo* hepatic lipogenesis. Since lipid export and beta oxidation were not upregulated to offset the large influx of dietary lipids, the disproportionate rate of lipid import to export and utilization is most likely to be the cause of the apparent hepatic lipid accumulation. It is important to note that the aim of this study was to find the role of methylation in hepatic lipid synthesizing genes. Our data suggest that this decrease in lipid synthesizing genes due to a post-weaning HFD is in a positive relationship with overall decreased gene methylation along the gene body of both ACC1 and GPAM.

In the second model, in which we looked at a maternal HFD, the HF/HF group showed an overexpression of FASN and GPAM, which provided us the physiological enzymatic mechanism for the apparent hepatic lipid accumulation. Since lipid accumulation requires an increase in available fatty acid and triacylglycerol production, the increase mRNA expression of FASN and GPAM provides the most plausible mechanism. The increased mRNA expression of GPAM was linked to an overall increase in gene body methylation; with no apparent differences in the promoter region of the gene. From the MEDIP-SEQ data, we were able to identify four different locations that showed increased methylation along the gene body for the HF/HF group, while the C/HF group only had one peak increase (Figures 4-9 & 4-10).

In order to validate the methylation patterns, we observed in the MEDIP-SEQ data, we performed a MSP with primers along six different locations on the gene body, which included both intronic and exonic positions. Although we saw increased methylation for the HF/HF group in all six locations, the following two specific locations showed a

significant increase for the HF/HF group: Intron 1 +7200 base pairs and Exon 21+59500 base pairs (Figure 4-11).

These data suggest that the increase in mRNA expression is in concordance with an overall increase in gene body methylation. As shown in the literature review and our hypothesis, this seems to contradict the concept of increased methylation leading to decreased gene expression. However, a few studies did indeed show that even on promoter methylation, increased methylation leads to increased gene expression [1,5,8]. Aside from the few studies that show a positive relationship between gene expression and methylation and the majority studies that show a negative relationship, the status on gene body methylation is unclear; especially when looking at the effects of a HFD on hepatic metabolic pathways.

To highlight this uncertainty in the role of gene body methylation, an *in vitro* study done by Shenker et al., showed that in breast cancer cells that are estrogen receptor alpha positive (ESR1+) there is a significantly higher percentage of methylation along the gene body than in ESR- cells. More specifically, they found the following three locations along the gene body to have a significantly increased percentage of methylation: +38810, +150393, and +204185 base pairs from the TSS [16]. These data seem to suggest and confirm our own results in that increased gene body methylation leads to increased gene expression. Shenker et al. then treated those cells with a demethylating agent, decitabine, which did indeed significantly reduce methylation levels. When comparing the differences between the decitabine treated cells to the C cells within ESR1+ cells, there was indeed a decrease in ESR1 gene expression. However, when treating ESR1- cells with decitabine and significantly reducing gene body methylation there was a stark increase

in gene expression; almost 20-fold [16]. This apparent paradox does not make clear what the exact role is regarding gene body methylation.

Another *in vitro* study that adds more information to this picture shows that demethylating the gene body of TWIST1 in gastric cancer cells leads to an increase in its gene expression. More specifically, Sakamoto et al. were able to find that the methylation of the exon 1 region was linked to the silencing of the TWIST1 expression even more so than the methylation of its promoter [26]. Brenet et al. confirmed this same result using a genome-wide analysis of DNA methylation and gene expression. They concluded that the first region in exon 1 is more tightly linked to the silencing of the gene expression than even promoter methylation [27].

These studies seem to suggest a significant role of gene body methylation in gene expression, as well as an apparent silencing role between the first exon and increased methylation. However, our study is the first to show increased methylation at the first intron and last exon in a positive relationship with mRNA expression for GPAM. Although we see a different metabolic physiology between similar enzymes due to the two inherently different models, the general gene body methylation patterns seem to maintain consistently in both models. Furthermore, our studies suggest that a HFD can either program or alter transcriptional gene expression of lipid synthesizing genes by altering gene body methylation in a positive relationship.

Tables & Figures

Figure 3-1. Experimental Design

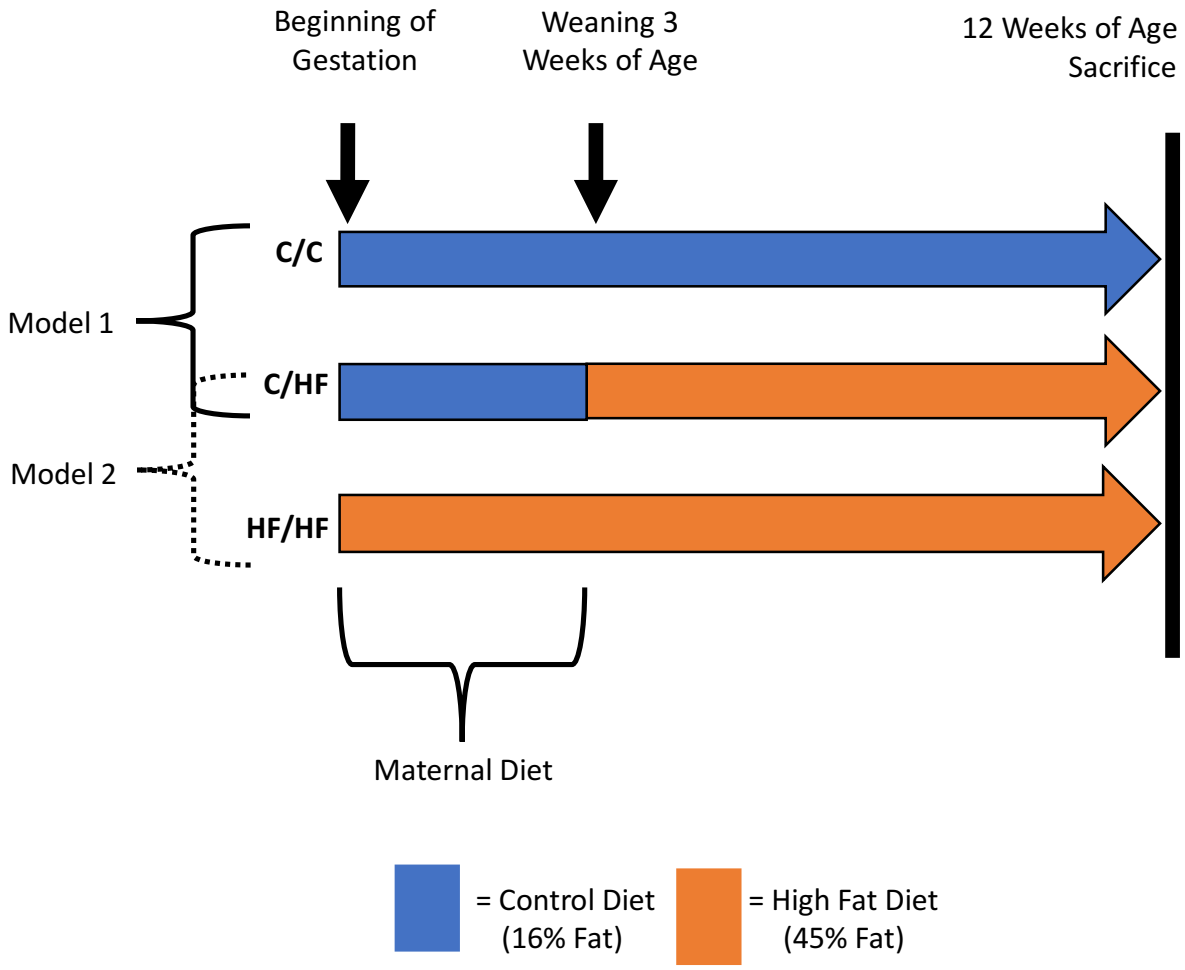
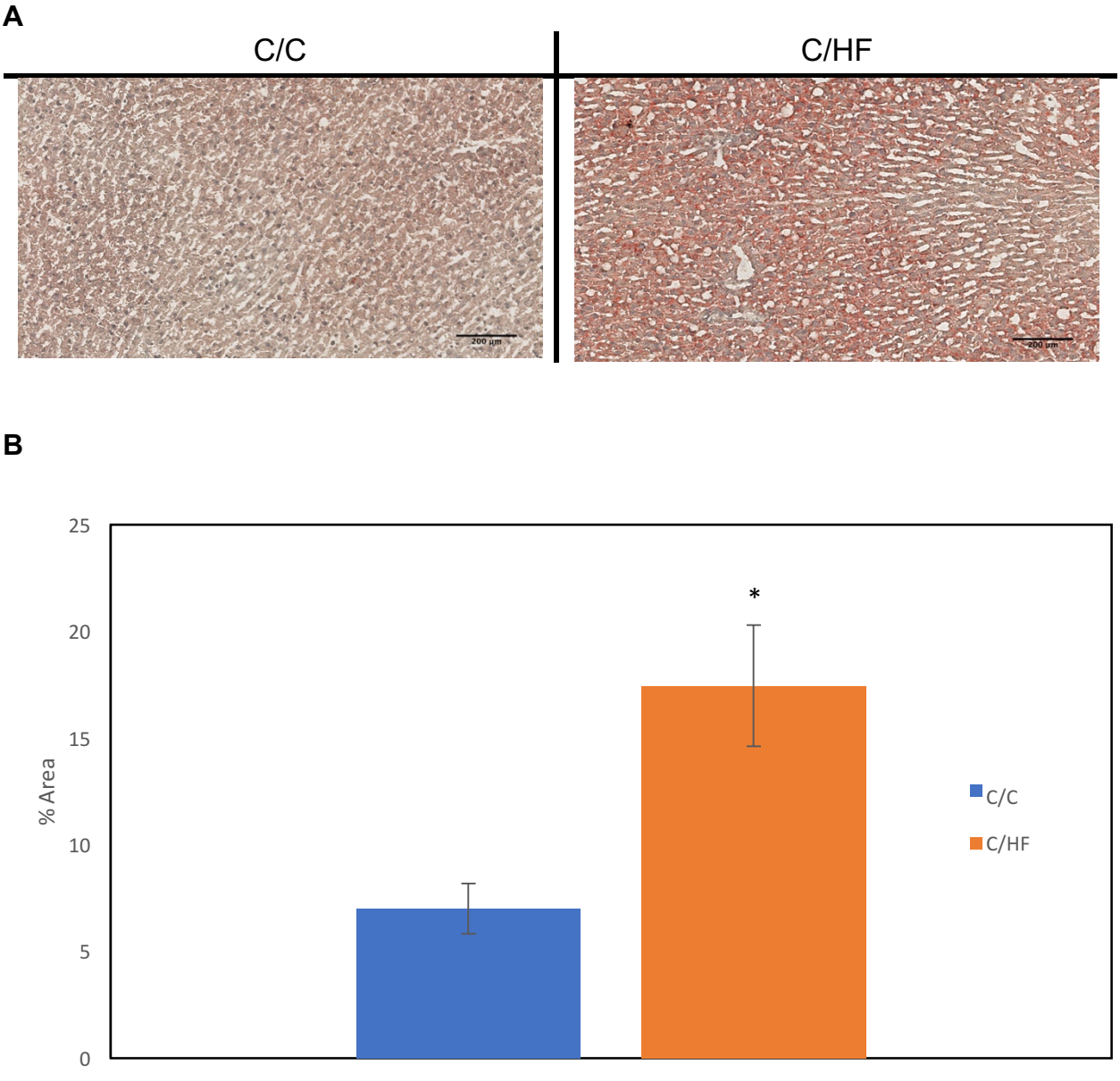


Table 4-1. mRNA Expression of Hepatic Metabolic Genes[1,2]

Gene Function	Gene	C/C	C/HF	HF/HF
TF of PEPCK	CEBPb	0.87 ± 0.131	1.17 ± 0.135	1.03 ± 0.085
	SREBP1-c	0.66 ± 0.055	0.91 ± 0.104	1.01 ± 0.105
GNG	G6Pase	**0.88 ± 0.176**	0.40 ± 0.089	0.75 ± 0.152
	FBP1	0.94 ± 0.161	0.82 ± 0.076	0.83 ± 0.065
	PEPCK	0.71 ± 0.056	0.63 ± 0.060	0.68 ± 0.132
Glycolysis	PFK1	0.80 ± 0.057	0.89 ± 0.067	0.98 ± 0.056
FA Synthesis	ACC1	**1.24 ± 0.118**	0.54 ± 0.097	0.68 ± 0.064
	FASN	**0.62 ± 0.087**	0.23 ± 0.049	**0.47 ± 0.062**
FA Oxidation	CPT1a	0.64 ± 0.067	0.86 ± 0.172	0.84 ± 0.072
TAG Synthesis	DGAT1	0.83 ± 0.081	0.71 ± 0.076	0.83 ± 0.066
	GPAM	**0.68 ± 0.136**	0.29 ± 0.037	**0.57 ± 0.056**
	AGPAT6	0.86 ± 0.065	0.88 ± 0.032	0.80 ± 0.051
Lipid Export	MTTP	1.13 ± 0.520	1.16 ±0.265	N/A

[1]All values are means ± SEM, n=8. Bolded values indicate a statistical significance when compared to C/HF.

Figure 4-1. Histological Assay[1]

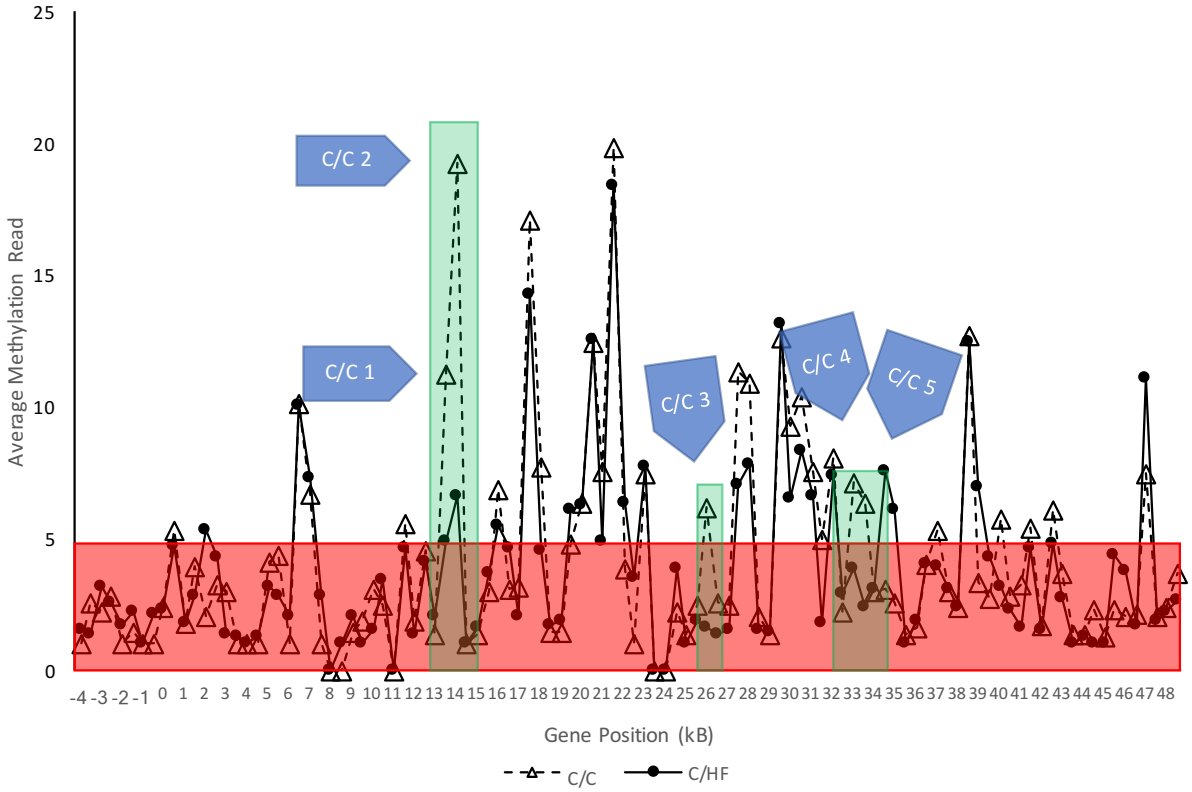


[1]Histological Assay Includes Oil Red O Staining and Quantification of Livers of Male Offspring Fed a HFD or C Diet. * Indicates a P-value < 0.05.

Figure 4-1. A) Oil Red O Staining: Red Color Indicates Lipid Deposition.

Figure 4-1. B) Quantification of Lipid Deposition

Figure 4-2. Average Methylation Read for Acetyl CoA Carboxylase 1: Promoter to 48 kB[1,2,3,4]



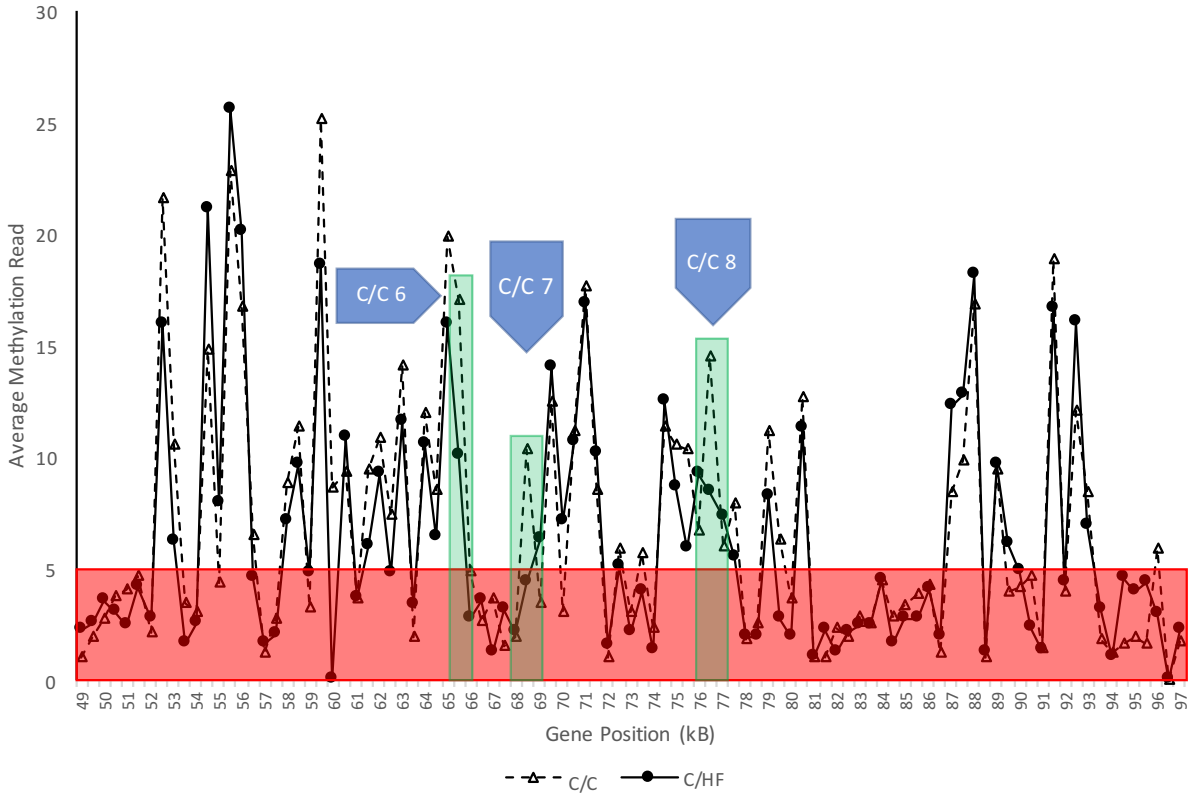
[1]Methylation Data obtained via MEDIP-SEQ-SEQ.

[2]Red Box Indicates Background Noise of MEDIP-SEQ Technique: Values that Fall Under 5 are Not Considered for Analysis.

[3]Green Boxes Indicate Highlighted Differential Methylated Peak Regions.

[4]Blue Boxes Indicate a Running Count of Methylation Peak Increases for the C/C Group.

Figure 4-3. Average Methylation Reads for Acetyl CoA Carboxylase 1: 49-97 kB[1,2,3,4]



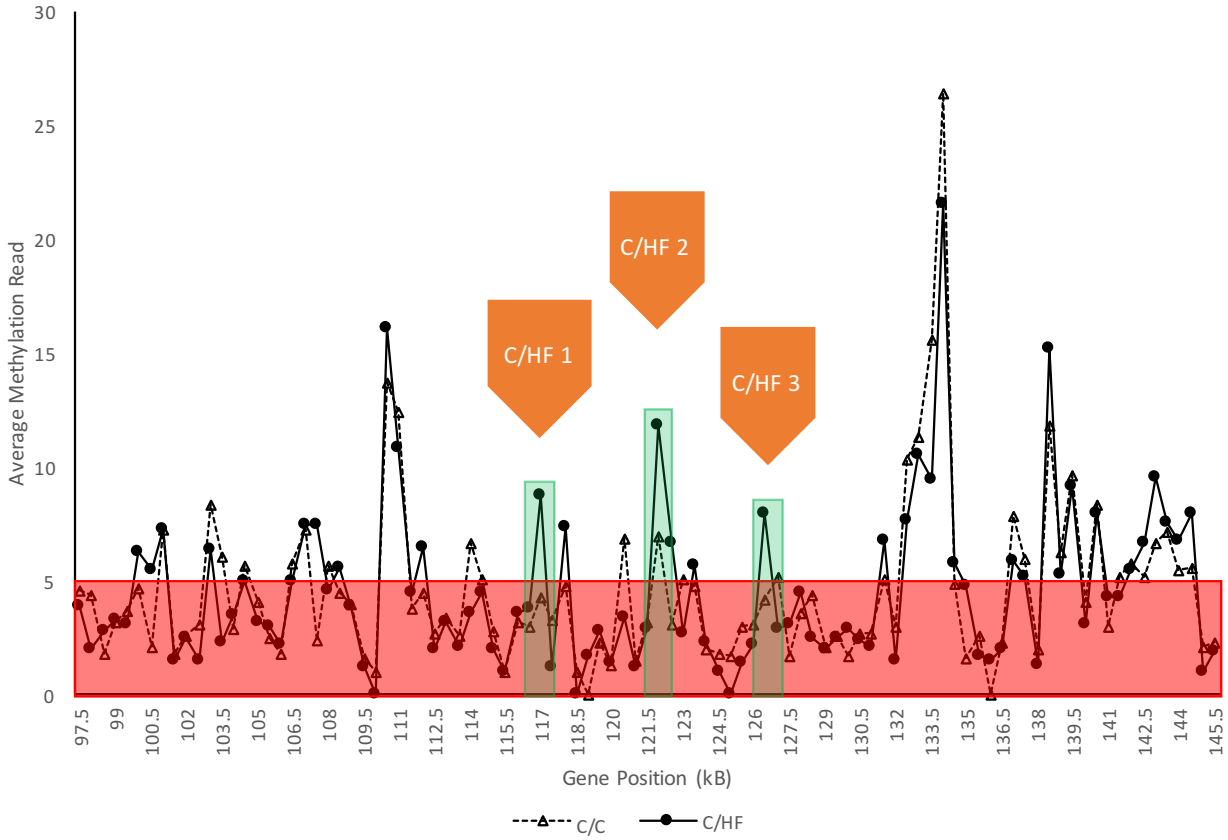
[1]Methylation Data obtained via MEDIP-SEQ.

[2]Red Box Indicates Background Noise of MEDIP-SEQ Technique: Values that Fall Under 5 are Not Considered for Analysis.

[3]Green Boxes Indicate Highlighted Differential Methylated Peak Regions.

[4]Blue Boxes Indicate a Running Count of Methylation Peak Increases for the C/C Group.

Figure 4-4. Average Methylation Reads for Acetyl CoA Carboxylase 1: 97.5-145.5 kB[1,2,3,4]



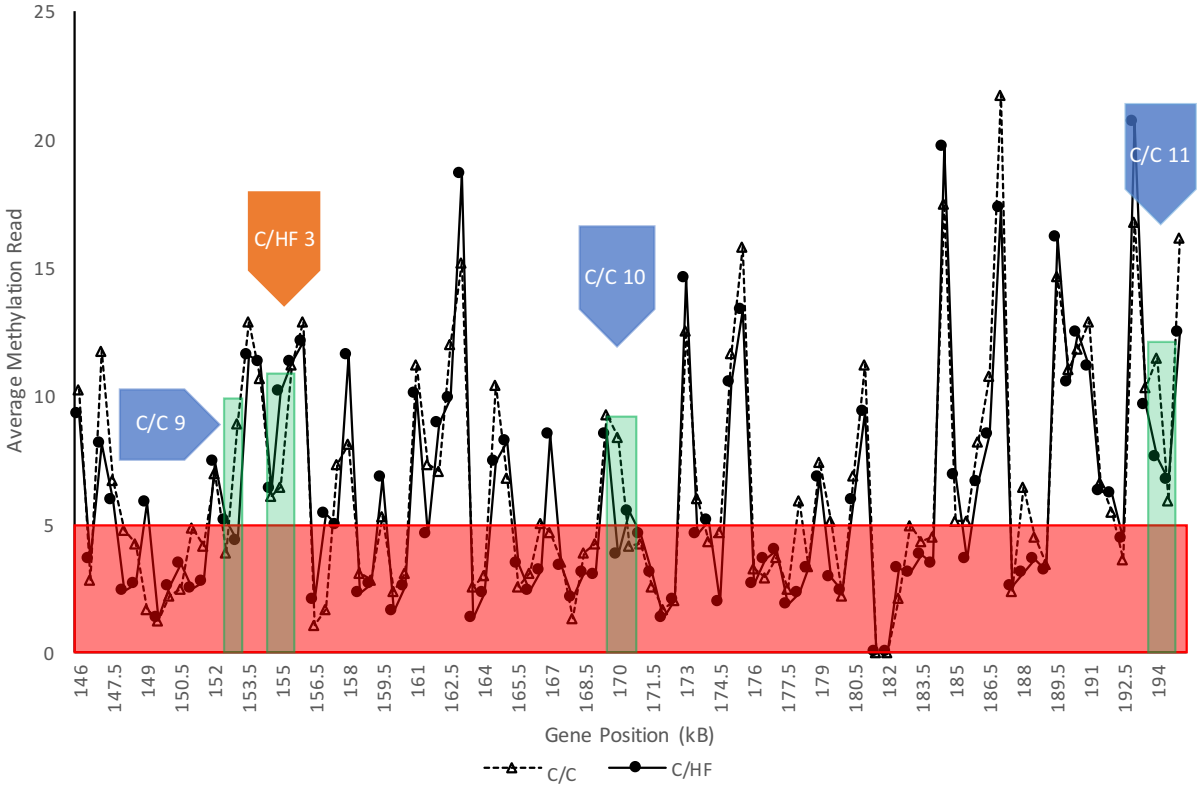
[1]Methylation Data obtained via MEDIP-SEQ.

[2]Red Box Indicates Background Noise of MEDIP-SEQ Technique: Values that Fall Under 5 are Not Considered for Analysis.

[3]Green Boxes Indicate Highlighted Differential Methylated Peak Regions.

[4]Orange Boxes Indicate a Running Count of Methylation Peak Increases for the C/HF Group.

Figure 4-5. Average Methylation Reads for Acetyl CoA Carboxylase 1: 146-194 kB[1,2,3,4,5]



[1]Methylation Data obtained via MEDIP-SEQ.

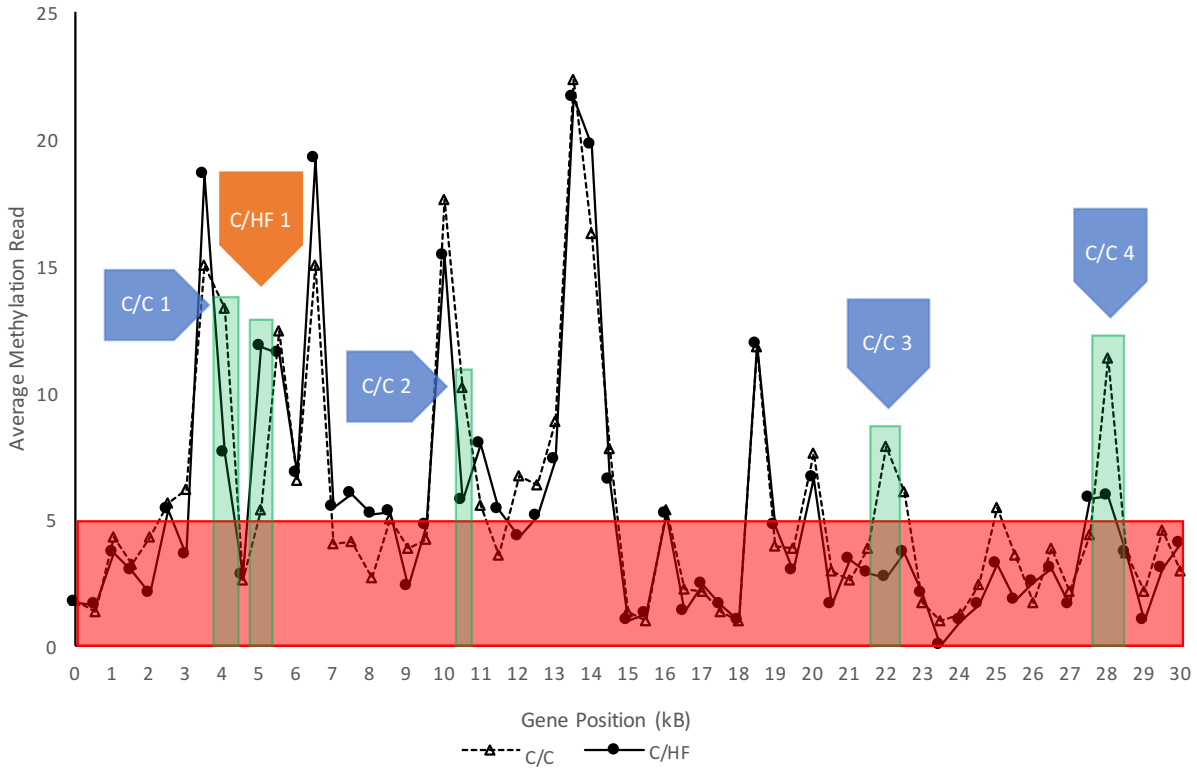
[2]Red Box Indicates Background Noise of MEDIP-SEQ Technique: Values that Fall Under 5 are Not Considered for Analysis.

[3]Green Boxes Indicate Highlighted Differential Methylated Peak Regions.

[4]Blue Boxes Indicate a Running Count of Methylation Peak Increases for the C/C Group.

[5]Orange Boxes Indicate a Running Count of Methylation Peak Increases for the C/HF Group.

Figure 4-6. Average Methylation Reads for Glycerol-3-Phosphate Acyltransferase: TSS to 30 kB[1,2,3,4,5]



[1]Methylation Data obtained via MEDIP-SEQ.

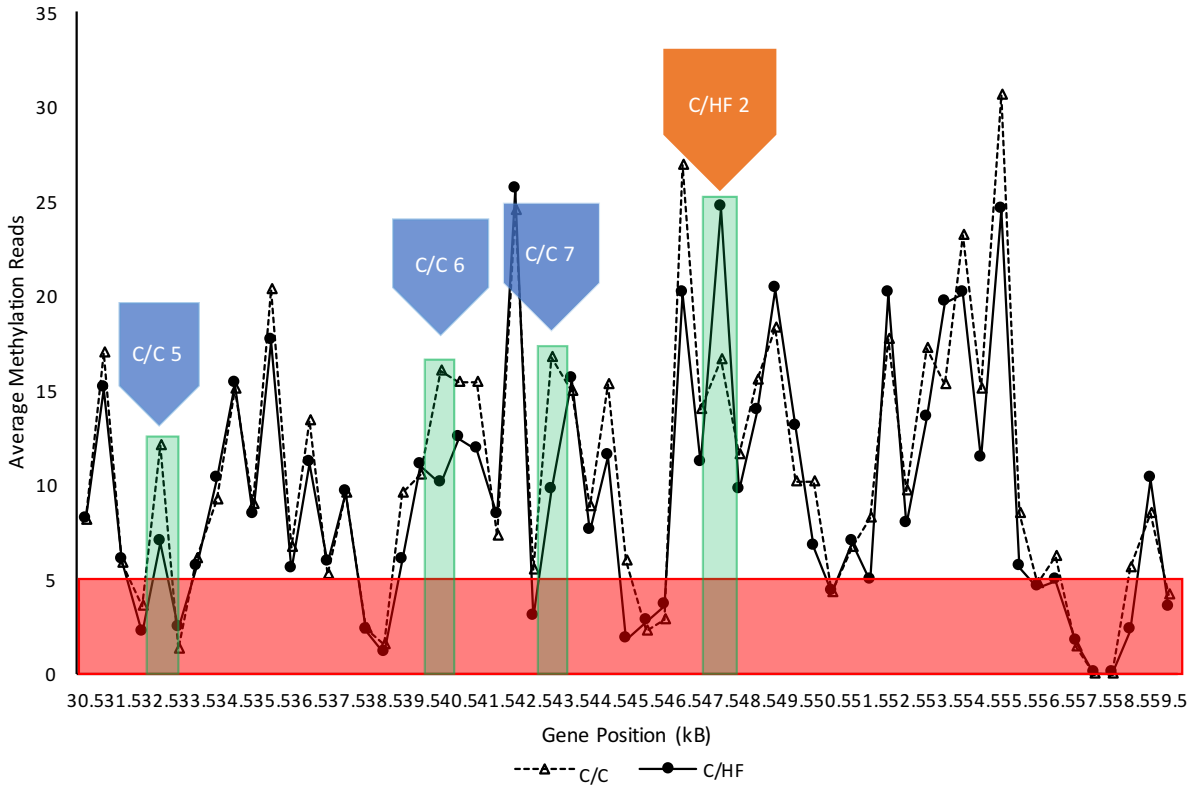
[2]Red Box Indicates Background Noise of MEDIP-SEQ Technique: Values that Fall Under 5 are Not Considered for Analysis.

[3]Green Boxes Indicate Highlighted Differential Methylated Peak Regions.

[4]Blue Boxes Indicate a Running Count of Methylation Peak Increases for the C/C Group.

[5]Orange Boxes Indicate a Running Count of Methylation Peak Increases for the C/HF Group.

Figure 4-7. Average Methylation Reads for Glycerol-3-Phosphate Acyltransferase: 30.5-59.5 kB[1,2,3,4,5]



[1]Methylation Data obtained via MEDIP-SEQ.

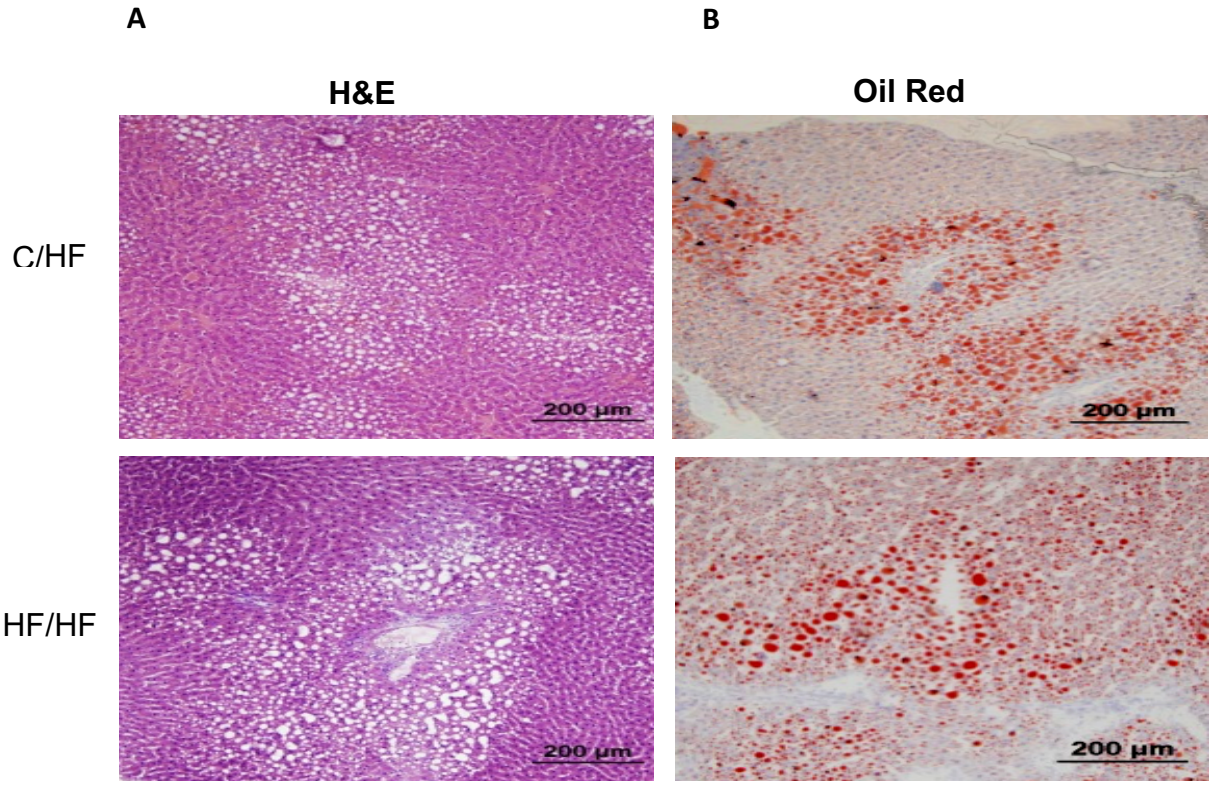
[2]Red Box Indicates Background Noise of MEDIP-SEQ Technique: Values that Fall Under 5 are Not Considered for Analysis.

[3]Green Boxes Indicate Highlighted Differential Methylated Peak Regions.

[4]Blue Boxes Indicate a Running Count of Methylation Peak Increases for the C/C Group.

[5]Orange Boxes Indicate a Running Count of Methylation Peak Increases for the C/HF Group.

Figure 4-8. Histological Assays[1,2,3]

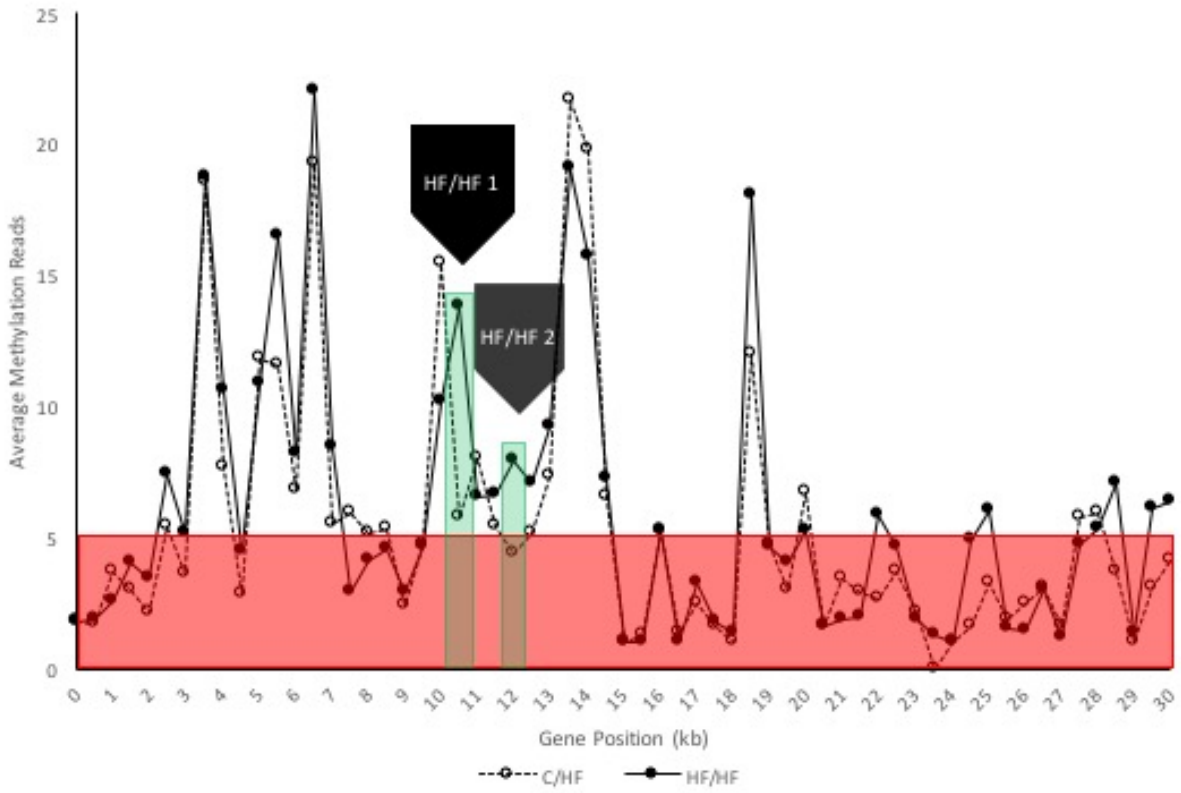


[1]Histological Assays Including H&E Stain (A) and Oil Red O Stain (B) of Livers of Male Offspring of Rat Dams Fed a HFD or C Diet.

[2]H&E Stain: White Circular Areas Indicate Hepatic Vacuolation

[3]Oil Red O Stain: Red Circular Areas Indicate Lipid Deposition

Figure 4-9. Average Methylation Reads for Glycerol-3-Phosphate Acyltransferase: TSS to 30 kB[1,2,3,4]



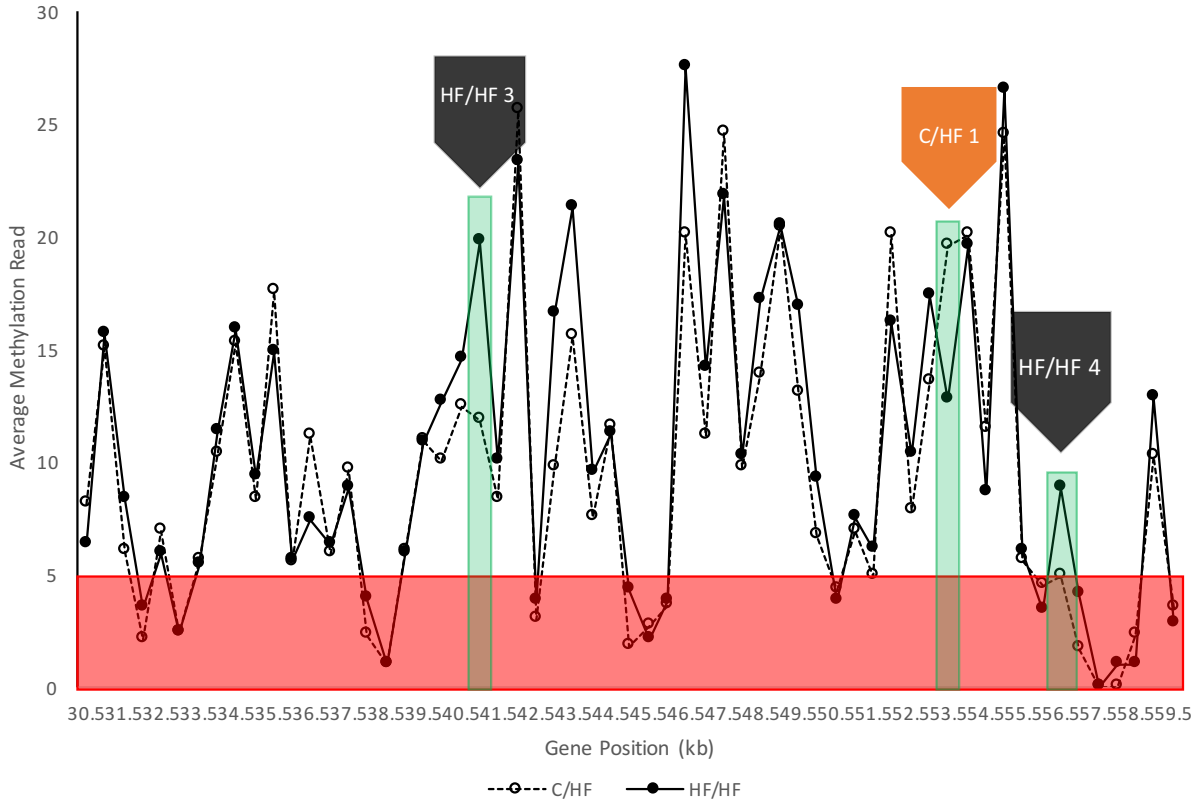
[1]Methylation Data obtained via MEDIP-SEQ.

[2]Red Box Indicates Background Noise of MEDIP-SEQ Technique: Values that Fall Under 5 are Not Considered for Analysis.

[3]Green Boxes Indicate Highlighted Differential Methylated Peak Regions.

[4]Black Boxes Indicate a Running Count of Methylation Peak Increases for the HF/HF Group.

Figure 4-10. Average Methylation Reads for Glycerol-3-Phosphate Acyltransferase: 30.5-59.5 kB[1,2,3,4,5]



[1]Methylation Data obtained via MEDIP-SEQ.

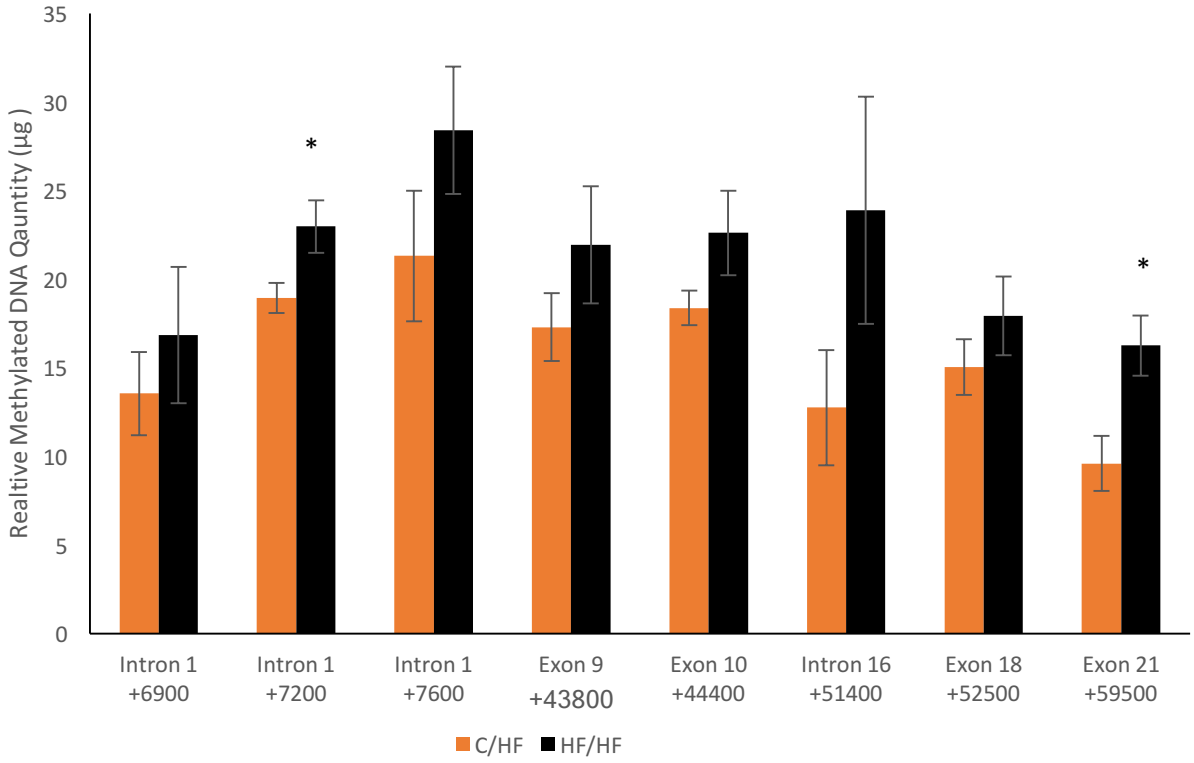
[2]Red Box Indicates Background Noise of MEDIP-SEQ Technique: Values that Fall Under 5 are Not Considered for Analysis.

[3]Green Boxes Indicate Highlighted Differential Methylated Peak Regions.

[4]Orange Box Indicates a Running Count of Methylation Peak Increase for the C/HF Group.

[5]Black Boxes Indicate a Running Count of Methylation Peak Increases for the HF/HF Group.

Figure 4-11. Methylation Levels Along Gene Body of GPAM[1,2,3]



[1]All values are means ± SEM, n=9 for C/HF and n=7 for HF/HF. * indicates a P-value < 0.05.

[2]Data obtained via MSP.

[3]Numbers Along X-axis Represent the Base Pair Location Along the Gene Relative to the TSS.

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